METHODS FOR DETECTING AND QUANTIFYING SPECIFIC MICROORGANISMS

DESCRIPTION

Cross Reference To Related Applications

[Para 1] The present application claims the benefit of U.S. Provisional Application Ser. 60/481312 filed Aug. 29, 2003 entitled "Method for the Detection of Microorganisms in Animal Feed," incorporated herein by reference. The present application is related to Disclosure Document No. 529733, received April 15, 2003, entitled "Analyzing Probiotics in Animal Feed".

Field of the Invention

[Para 2] The invention relates to materials and methods useful for the detection and quantification of specific microorganisms in a sample of material. The methods include the culturing of microorganisms and use of oligonucleotide primers to detect specific microorganisms of interest.

Description of the Related Art

[Para 3] Microorganisms are often added to animal feed in order to provide nutritional supplements, to improve digestion, to increase uptake of desirable nutrients, to compete with undesirable or harmful microorganisms, and various other reasons. Typically the microorganisms are added to the animal feed at the location where the animal feed is to be consumed by the animals, such as at a feedlot or dairy. See, for example, Ware et al. U.S. patent No. 5,534,271 issued July 9, 1996 entitled "Process for Improving the Utilization of Feedstuffs by Ruminants," incorporated herein by reference, and Garner et al. U.S. Patent No. 5,529,793 issued Jun. 25, 1996 entitled "Composition for Improving the Utilization of Feedstuffs by Ruminants," incorporated herein by

reference. The terms "probiotic" and "direct fed microbials" (DFM) are often used in reference to beneficial microorganisms that are added to animal feed.

[Para 4] One of the challenges involved is the need to verify the presence of the added microorganisms, and to quantify their concentration. Most existing methods rely on direct or indirect culturing of samples obtained from treated feed. These methods are often compromised by the presence of other microorganisms, often in significantly higher concentrations. Additionally, many microorganisms appear similar when cultured on traditional media, further complicating their identification and quantification.

[Para 5] Thus, there exists a need for improved methods of analyzing a sample of material, ideally allowing the verification of the presence of a particular strain of microorganism.

Summary of Invention

[Para 6] Methods combining the culturing of samples and use of oligonucleotide primers are disclosed. The oligonucleotide primers can be used in direct detection methods, or can be used in methods such as the Polymerization Chain Reaction (PCR).

[Para 7] In accordance with one aspect, the invention provides a method of quantifying a presence of a specific kind of microorganism in a sample of material. The method includes: (a) culturing the sample under conditions suitable for growth of cultures of the specific kind of microorganism; (b) using at least one oligonucleotide to detect the presence or absence of the specific kind of microorganism in respective portions of the cultured sample; and (c) quantifying the presence of the specific kind of microorganism in the sample of material from the detected presence or absence of the specific kind of microorganism in the respective portions of the cultured sample.

[Para 8] In accordance with another aspect, the invention provides a method of quantifying a presence of a specific kind of microorganism in a sample of material. The method includes: (a) dividing the sample into multiple portions; (b) culturing each portion of the sample under conditions suitable for growth

of the specific kind of microorganism; (c) performing a polymerase chain reaction process by reacting each cultured portion of the sample successively with two oligonucleotide primers that selectively hybridize with nucleic acid of the specific kind of microorganism to produce a respective reaction product from each cultured portion of the sample; (d) detecting the presence or absence of a reaction product having a characteristic length from the reaction of each cultured portion of the sample; and (e) quantifying the presence of the specific kind of microorganism in the sample of material from the detected presence or absence of a reaction product having a characteristic length from the reaction of each cultured portion of the sample.

Brief Description of Sequences

[Para 9] The sequence listings following the detailed description below form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these sequences in combination with the detailed description of specific embodiments presented herein.

[Para 10] SEQ ID NO:1 is oligonucleotide PCR primer Lacto G5R (18 nt).

[Para 11] SEQ ID NO:2 is oligonucleotide PCR primer LA51 specific G4R (18 nt).

[Para 12] SEQ ID NO:3 is an operon ITS target rRNA sequence to which SEQ ID NO:2 hybridizes.

Brief Description of Drawings

[Para 13] Figure 1 is a summary in flow chart form of a method for quantifying the presence of a specific microorganism in a sample of material.

Detailed Description

[Para 14] Typical methods of verifying and quantifying the presence of a specific kind of microorganism rely on cultures made on petri dishes, resulting

in the calculation of a "plate count" or "cfu" (colony forming unit) count. These methods are inaccurate, and cannot distinguish between similar types of microorganisms that may appear visually similar or identical when growing on a petri dish.

[Para 15] Various embodiments of the instant invention use oligonucleotides to either directly or indirectly detect and quantify microorganisms in a sample of material.

[Para 16] Aspects of the instant invention relate to the use of PCR (polymerase chain reaction) methods to accurately verify and quantify the presence of a specific kind of microorganism in a sample of material. Other aspects of the instant invention relate to the hybridization of oligonucleotide primers to distinctive DNA or RNA sequences from one or more microorganisms of interest, followed by detection and/or quantification of the hybridized primers.

[Para 17] The methods of the present invention can be used to detect and quantify the presence of probiotic microorganisms. For example, the methods of the present invention can be used to detect and quantify a specific kind of probiotic microorganism in animal feed. The animal feed can generally be any type of animal feed. Examples of animal feed include dairy cattle feed, beef cattle feed, feedlot cattle, dog food, cat food, rabbit food, zoo animal food, cow feed, chicken feed, horse feed, pig feed, turkey feed, lamb feed, deer feed, buffalo feed, alligator feed, snake feed, and fish feed.

[Para 18] The probiotic microorganism can generally be any probiotic microorganism that is desirable to add to animal feed or to administer to an animal directly or by other means. Examples of such probiotic microorganisms include Bacillus subtilis, Bifidobacterium adolescentis, Bifidobacterium animalis, Bifidobacterium bifudum, Bifidobacterium infantis, Bifidobacterium longum, Bifidobacterium thermophilum, Lactobacillus acidophilus, Lactobacillus agilis, Lactobacillus alactosus, Lactobacillus alimentarius, Lactobacillus amylophilus, Lactobacillus amylovorans, Lactobacillus amylovorus, Lactobacillus animalis, Lactobacillus batatas, Lactobacillus bavaricus, Lactobacillus bifermentans, Lactobacillus bifidus, Lactobacillus brevis, Lactobacillus buchnerii, Lactobacillus bulgaricus,

Lactobacillus catenaforme, Lactobacillus casei, Lactobacillus cellobiosus, Lactobacillus collinoides, Lactobacillus confusus, Lactobacillus coprophilus, Lactobacillus coryniformis, Lactobacillus corynoides, Lactobacillus crispatus, Lactobacillus curvatus, Lactobacillus delbrueckii, Lactobacillus desidiosus, Lactobacillus divergens, Lactobacillus enterii, Lactobacillus farciminis, Lactobacillus fermentum, Lactobacillus frigidus, Lactobacillus fructivorans, Lactobacillus fructosus, Lactobacillus gasseri, Lactobacillus halotolerans, Lactobacillus helveticus, Lactobacillus heterohiochii, Lactobacillus hilgardii, Lactobacillus hordniae, Lactobacillus inulinus, Lactobacillus jensenii, Lactobacillus jugurti, Lactobacillus kandleri, Lactobacillus kefir, Lactobacillus lactis, Lactobacillus leichmannii, Lactobacillus lindneri, Lactobacillus malefermentans, Lactobacillus mali, Lactobacillus maltaromicus, Lactobacillus minor, Lactobacillus minutus, Lactobacillus mobilis, Lactobacillus murinus, Lactobacillus pentosus, Lactobacillus plantarum, Lactobacillus pseudoplantarum, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactobacillus rogosae, Lactobacillus tolerans, Lactobacillus torquens, Lactobacillus ruminis, Lactobacillus sake, Lactobacillus salivarius, Lactobacillus sanfrancisco, Lactobacillus sharpeae, Lactobacillus trichodes, Lactobacillus vaccinostercus, Lactobacillus viridescens, Lactobacillus vitulinus, Lactobacillus xylosus, Lactobacillus yamanashiensis, Lactobacillus zeae, Pediococcus acidlactici, Pediococcus pentosaceus, Streptococcus cremoris, Streptococcus discetylactis, Streptococcus faecium, Streptococcus intermedius, Streptococcus lactis, Streptococcus thermophilus, and Escherichia coli. Another group of lactate utilizing microorganisms include Propionibacterium freudenreichii, Propionibacterium shermanii, Propionibacterium jensenii, Propionibacterium acidipropionici, Propionibacterium thoenii, Propionibacterium, Megasphaera elsdenii, Selenomonas ruminatium, and Peptostreptococcus asaccharolyticus. One specific example of a probiotic microorganism is a species of Lactobacillus such as Lactobacillus acidophilus or Lactobacillus strain LA51. Strain LA51 is a naturally occurring strain. A supply of the strain LA51 has been maintained by Professor Stanley Gilliland at the University of Oklahoma, and samples have been offered under license from the University of Oklahoma.

[Para 19] The methods disclosed herein can also be used to detect and quantify the presence of harmful or undesirable microorganisms in animal feed, food for human consumption, soil, water, plants, animal hide and skin, the digestive track of animals, manure, and feces. Harmful or undesirable microorganisms include Escherichia spp., Salmonella spp., Shigella spp., Campylobacter spp., Clostridium spp., Mycobacterium spp., Yersinia spp., Bacillus spp., Vibrio spp., Staphylococcus spp., Streptococcus spp., Aeromonas spp., Klebsiella spp., Entrobacter spp., Proteus spp., Citrobacter spp., Aerobacter spp., and Serratia spp.

[Para 20] The culturing step is preferably performed under conditions favorable for growth of the microorganism of interest. Different microorganisms have different optimal temperature, media, and pH conditions. For example, Lactobacillus acidophilus grows well in an anaerobic environment at about 35 °C and a pH of about 5.5.

[Para 21] The oligonucleotide primers are preferably selected to hybridize to a unique specific nucleic acid sequence present in microorganism of interest. The specific nucleic acid sequence is preferably not present in other microorganisms commonly found in the sample of material. The specific primer length and sequence depend on the nucleic acid sequence. Generally, the oligonucleotide primers are about 10 nucleotides to about 25 nucleotides in length. For example, the primers can be 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more nucleotides in length, up to at least 35 nucleotides.

[Para 22] Two oligonucleotide primers are used in the PCR process. The two PCR primers can have the same length or can have different lengths. PCR primers preferably do not have significant secondary structure that could interfere with hybridization to the specific nucleic acid sequence. Also, the PCR primers preferably do not have considerable repeats of sequences that may lead to false hybridization. It is also preferable that the two PCR primers used in the PCR reaction sequence do not have regions of complementarity that could lead to their hybridizing to each other rather than to the specific nucleic acid sequence.

[Para 23] Reaction products of the PCR reaction sequence can be analyzed by a variety of well known molecular biological methods. These methods include agarose gel electrophoresis, polyacrylamide gel electrophoresis, and liquid chromatography. These methods may include imaging techniques such as microscopic imaging of electrophoresis results.

[Para 24] The quantification of a specific microorganism in a sample of material can be compared to samples of the material dosed with known quantities of the specific microorganism. The quantification of a specific microorganism in a sample of material also can be compared to control or "blank" samples. For example, the quantification of a specific probiotic mircoorganism in animal feed samples can be compared to the quantification of the specific probiotic mircoorganism in samples dosed with known quantities of the specific probiotic microorganism, and also compared to the quantification of the specific probiotic mircoorganism in control "blank" samples. This comparison can be qualitative, resulting in a "yes/no" result, or quantitative, resulting in calculation of the concentration of specific kind of microorganism in the sample of material, such as the concentration of a probiotic microorganisms present in animal feed.

[Para 25] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the scope of the invention.

[Para 26] EXAMPLES

Example 1: Sampling of animal feed

[Para 27] An important first step in an analysis of animal feed is the obtaining of representative samples. While many suitable methods may be designed, the following has been found to be effective.

[Para 28] Ten samples of about 500 grams each are obtained. The samples are placed in sterile plastic bags, and are sealed. The bags are marked regarding the date and time that the sample was taken, and the amount of probiotic added to the animal feed (typically per ton of feed). Samples are obtained randomly, and from materials dispersed within the feed. For example, a 10,000 pound load of feed can be sampled once every 1,000 pounds for a total of 10 samples. If the probiotic is known or suspected of being sensitive to light, heat, or air, then the samples should be obtained from the "inside" of the feed pile.

[Para 29] The same number of control samples can be taken from the same type of feed that was not treated with the probiotic. The control samples are useful for determining background levels of organisms. Care should be taken with the sampling and handling equipment so as to not contaminate the control samples.

[Para 30] Samples can be stored in an insulated cooler, and delivered to a testing laboratory as soon as possible. The samples can be placed in a transport media, such as LBS broth, and maintained at a cool temperature, such as 4 degrees Centigrade, sufficient to inhibit growth of microorganisms, during transport to the testing laboratory.

Example 2: Media

[Para 31] Liquid or solid media should be selected to be suitable for growth of the probiotic. For example, when assaying for the probiotic Lactobacillus LA51, LBS broth and LBS agar can be used according to the manufacturer's protocols. LBS is commercially available from a wide array of suppliers including Sigma-Aldrich (St. Louis, MO) and Alpha Biosciences (Baltimore, MD). LBS obtained from Alpha Biosciences has a pH of 5.5 ±0.2 at 25 °C and contains the following components: casein digest peptone (10.0g/l), dextrose (20.0g/l),

yeast extract (5.0g/l), sodium acetate (25.0g/l), monopotassium phosphate (6.0g/l), Tween 80 (1.0g/l), ferrous sulfate (0.034g/l), ammonium citrate (2.0g/l), magnesium sulfate (0.575g/l), manganese sulfate (0.12g/l), and agar (for solid media, 15.0g/l).

Example 3: LBS plating of probiotics

[Para 32] Ten grams of sample is added to 90 ml of 0.1% peptone in distilled water. The mixture is shaken in a mixing cylinder 30 times. The mixture is allowed to stand for 10 minutes. This is the -1 dilution.

[Para 33] Multiple additional serial dilutions are performed as needed to provide a reasonable number of colonies growing on an LBS plate to count. For example, dilutions of -1, -2, -3, -4, -5, and -6 can be made. Depending on the size of the plate used, a small volume of the dilution is spread evenly across the surface of the plate for culturing. Typically, 0.1 to 1 ml of liquid is used. Plates can be prepared singly or in replicates for enumeration.

[Para 34] Plates are covered, and incubated in an anaerobic environment for 48 hours at 35 °C. The counts on the plates are determined. Typically, between 30 and 300 counts per plate is reasonable. Multiple colonies from the plate can be examined microscopically. Typically about five colonies per plate are examined. The color and shape of the colony is recorded. For the probiotic Lactobacillus LA51, the colonies should be white and round in appearance.

[Para 35] A slide can be prepared for a gram stain assay. LA51 colonies evaluated should be gram positive, and the organisms should appear as rounded rods.

Example 4: Addition of standards to animal feed

[Para 36] Control feed is autolyzed, and allowed to cool to room temperature. The same concentration of probiotic is added to the cooled feed as was added to the treated samples. The probiotics are allowed to soak in the feed for 10 minutes. Ten grams of treated feed is added to 90 ml of 0.1% peptone in

distilled water, as described in the previous Example. Serial dilutions, incubation, plating, and analysis of these samples are performed in the same manner as described in Example 3.

Example 5: Culturing of probiotics from treated feed

[Para 37] 2.25 liters of 0.1% peptone in distilled water is added to a mixing cylinder. Ten portions of 25 grams feed is added, one from each of the ten sample bags. A mixing ball is added, and the cylinder is shaked for 60 seconds. This is the -1 dilution. The mixture is allowed to stand for 10 minutes. Serial dilutions, incubation, plating, and analysis of these samples are performed in the same manner as described in Example 3.

Example 6: Culturing of probiotics from control feed

[Para 38] The procedure from Example 3 is used with the control feed samples. This gives an indication of the background microorganisms present in untreated feed.

Example 7: PCR analysis of samples

[Para 39] The previous Examples can be used to obtain a "presumed" cfu count of probiotics present in animal feed. However, many organisms may appear similar or identical to the probiotic, resulting in over-counting of probiotic cfus. Also, the presence of the probiotic or other component of the animal feed treatment may stimulate or inhibit growth of non-probiotic organisms, further complicating the analysis. The use of the polymerase chain reaction (PCR) analysis technique provides clear evidence of the presence of a particular probiotic in the animal feed samples.

[Para 40] PCR assays for the presence (or absence) of a particular DNA sequence in a sample. PCR does not distinguish between DNA obtained from a living organism and DNA obtained from a dead or non-viable organism. Accordingly, the serial dilution cultures described in the previous Examples

can be used to amplify the "signal" obtained from living organisms in the samples. The quantity of non-viable organisms would be a small percentage of the viable organisms after the incubation phase, and would therefore be of minor consequence in the subsequent PCR analysis.

[Para 41] PCR can be performed on specific colonies growing on plates, or on liquid culture samples. A small quantity of a colony can be added to a PCR reaction using a toothpick or the tip of a micropipette. A small volume of liquid culture (e.g. 1 microliter) can be added to the PCR reaction directly. Too much of either type of sample may inhibit the PCR reaction. A sample to be added to a PCR reaction can be centrifuged and washed in distilled water in order to eliminate fermentation products.

Example 8: Preparation of PCR reaction samples

[Para 42] A DNA sequence from the probiotic is selected to be amplified using PCR. Ideally, the particular DNA sequence would be unique among the microorganisms commonly found in animal feed, and would therefore act as a distinctive "marker" for the presence or absence of the probiotic in the sample. In this Example, the operon ITS target rRNA sequence was chosen (SEQ ID NO:3).

[Para 43] For each 25 microliter reaction, the following components are combined: 12.5 microliters HotstarTaq Master Mix (Qiagen, Inc., Valencia, CA), 1 microliter primer Lacto G4R (50 nanograms per microliter; 5'-AAC GCG GTG TTC TCG GTT -3' (SEQ ID NO:1)), 1 microliter primer LA51 specific (50 nanograms per microliter; 5'-CCT GCA CTT TAT CTA TCG-3' (SEQ ID NO:2)), and 9.5 microliters distilled water. Primer SEQ ID NO:1 was chosen as a generalized sequence matching Lactobacilli. (SEQ ID NO:1 is complementary to the reverse nucleotide sequence from nucleotides 563 to 546 in SEQ NO:3.) Primer SEQ ID NO:2 is designed to hybridize to an LA51 sequence on the internal transcribed spacer ("ITS") located between the 16S and 23S region of rRNA. (SEQ NO:2 is the nucleotide sequence from nucleotides 342 to 359 in SEQ ID NO:3.)

[Para 44] The sample (1 microliter liquid culture, or a small quantity of colony material) is added to the PCR reaction tube and mixed. Positive and negative control samples are also prepared.

[Para 45] The PCR reaction tubes are placed in a thermocycler PCR instrument, and processed using a suitable time and temperature program. For the above primers, the following program is effective: 32 cycles of (94 °C denaturing for 30 seconds, 54 °C annealing for 30 seconds, and 72 °C polymerizing for 1 minute), then 72 °C for 10 minutes, and storage at 4 °C.

[Para 46] PCR products are readily analyzed using horizontal agarose gel electrophoresis. A 1.75% agarose gel made in 1x TAE buffer containing 0.1 microliter per ml ethidium bromide can be used. For the above described PCR reaction, 8 microliters of reaction mixture is combined with 2 microliters of 5x loading buffer (containing bromphenol blue marker), and added into a well in the agarose gel. A size standard (e.g. phiX 174 DNA cut with restriction enzyme HaeIII) is added into one lane of the gel. The gel is run at 25-50 volts. Progress of the electrophoresis is monitored by visual inspection of the bromphenol blue band in the gel. DNA bands are visualized using a UV light source. PCR analysis of DNA from probiotic Lactobacillus LA51 using primers SEQ ID NOS:1 and 2 produces a single band of about 225 bp.

[Para 47] PCR reactions using various known concentrations of standards can be used to quantify the concentration of probiotic in the culture. This, combined with the degree of serial dilution, can be used to quantify the concentration of probiotic in the animal feed.

Example 9: Interpretation of assay results

[Para 48] Animal feed can be treated with probiotic Lactobacillus LA51 at 2.0 x 10 exp 10 to 2.6 x 10 exp 10 cfu/g. The following results are expected from using the methods described in the previous Examples. Most Probable Number ("MPN") is a method for estimating low concentrations of organisms based on observation of serial dilutions (Cochran, W. G. 1950. Estimation of bacterial densities by means of the "Most Probable Number." Biometrics 6:105-116;

James T. Peeler and Foster D. McClure; Bacteriological Analytical Manual, USFDA, 7th edition, 1992).

Sample	Plate Count	Most Probable Number
LA51 probiotic culture	2.4×10^{10} /g	$2.0 - 2.4 \times 10^{10}$ /g
Control feed	1×10^{3} /g - 1×10^{7} /g	0
Autolyzed (lab treated)	5×10^{4} /g – 1.6×10^{5} /g	5×10^{4} /g – 1.6×10^{5} /g
Treated feed	5×10^{4} /g – 1.6×10^{7} /g	5×10^{4} /g – 1.6×10^{5} /g

[Para 49] Control feeds containing LA51 are most likely contaminated.

Example 10: Exemplary assay results

[Para 50] Animal feed was treated with probiotic Lactobacillus LA51 at 2.0 x 10exp10 cfu/g. The probiotic was allowed to contact the feed for 5.5 hours prior to sampling. The following counts were determined, and were all found to be within the expected ranges.

	Background	Detected LA51	Expected LA51
Culture	0	2.4×10^{10}	2.0×10^{10}
Control feed	3.7×10^{5}	0	0
Autolyzed / treated	0	7.3×10^{4}	1.0×10^{5}
Treated feed	3.7×10^{5}	6.7×10^{4}	1.0×10^{5}

[Para 51] Next, samples were observed using a microscope and by gram staining

	Colonies observed	Microscopic	Gram Stain
Culture of LA51	5 round white	Round rods	Gram +
Control Feed	3 irregular/clear	Cocci	Gram -
	2 large white	Long rods	Gram +
Autolyzed / treated	5 round white	Round rods	Gram +
Treated feed	2 irregular/clear	Cocci	Gram -
	3 round white	Round rods	Gram +

[Para 52] The Control Feed and Treated Feed contained similar levels of presumptive LA51 counts and similar observed organisms. However, by observing amplified PCR products, only the Treated Feed contained LA51. About 43 percent of expected organisms were extracted from the feed by use of a mixing ball. This allowed for positive identification of LA51, and also assured a level within the expected range of content of organisms. While only 43% of expected organisms seems to be low, obtaining 100% of expected live organisms is somewhat unrealistic. Any recovery above 10% places the determination within the same logarithm of expected counts.

[Para 53] Although the above examples show the detection and quantification of a specific kind of microorganisms in animal feed, it should be understood that the methods employed in these examples can be adapted to detecting and quantifying specific microorganisms in samples of other kinds of material. The methods can be used for detecting and quantifying specific pathogens in animal feed, or detecting specific microorganism in manure or in the digestive track of an animal. The methods can be used for detecting and quantifying specific microorganisms in food, water, or air. The methods can be used for detecting and quantifying a specific microorganism having peculiar conditions for germination or transmission. The methods could also be useful for

detecting and quantifying a specific virulent or contagious natural or bioengineered mutation of an otherwise common or benign microorganism.

[Para 54] FIG. 1 shows a summary of a method employed in a number of the above examples. This method is suited for automated processing of a sample and quantification of low concentrations of a specific kind of microorganism of interest without the use of radioactive markers or probes. In a first step 101, a representative sample of material is taken. The sample should be taken so as to be representative of the bulk of the material to be used or consumed. For example, the sample is taken at or near the time and place where the bulk of the material is used or consumed, such as at a feedlot or dairy in the case of animal feed.

[Para 55] In step 102, the sample is diluted so that in a later step (107) a good number of cultured portions of the sample will have indications of the absence of the specific kind of microorganism of interest. Step 102 may be omitted if the initial concentration of the specific kind of microorganism is sufficiently low.

[Para 56] In step 103, the diluted sample is divided into multiple portions. In step 104, each portion of the diluted sample is cultured under conditions suitable for growth of the microorganism. In step 105, a PCR process is performed by successively reacting each cultured portion with two oligonucleotide primers that selectively hybridize with DNA of the microorganism.

[Para 57] The number of PCR amplification cycles to be used upon each cultured portion can be chosen by preparing standard samples each containing a small number of the microorganism per sample, and performing PCR amplification upon the standard samples using respective numbers of cycles spread over a wide range of cycles. There should be a minimum number of cycles at which a positive indication is obtained (by electrophoresis detection as in step 106). There may be a maximum number of cycles at which a positive indication is no longer valid. The number of cycles to be used upon each cultured portion should be a median between these minimum and maximum numbers. This calibration of the PCR process can also be done upon

standard samples prepared by adding known quantities of the microorganism to sterilized and unsterilized quantities of the material to be sampled, in order to adjust the number of PCR cycles to compensate for effects of the material to be sampled or competing microorganisms in the material to be sampled.

[Para 58] In step 106, electrophoresis is performed upon the PCR reaction product from each portion of the diluted sample to detect the presence or absence of a reaction product having a characteristic length.

[Para 59] In step 107, the most probable number of the specific kind of microorganism in the sample is determined by assuming that, for each portion of the diluted sample, the presence or absence of a PCR reaction product having the characteristic length indicates the presence or absence of at least one of the specific kind of microorganism. The number of portions of the diluted sample indicated as having at least one of the specific kind of microorganism is a lower bound to the number of the specific kind of microorganism in the portions of the sample prior to incubation.

[Para 60] By assuming that the specific kind of microorganism in the sample are randomly distributed among the sample portions, one can determine the most probable number of the specific kind of microorganism initially in the sample from the number of sample portions indicated as having at least one of the specific kind of microorganism. Moreover, confidence limits can be established that also take into account random variation of the sample from the bulk of the material from which the sample is taken.

[Para 61] For example, tables showing the most probable number of microorganisms and high and low 95% confidence limits given a particular number of positive indications for the cases of N=3, 5, 8, and 10 sample portions are published on the Internet web site of the Center for Food Safety & Applied Nutrition of the U.S. Federal Drug Administration (cfsan.fda.gov) in the Bacteriological Analytical Manual Online, January 2001, Appendix 2, Most Probable Number from Serial Dilutions, by Robert Blodgett. Data in the table for the case of N=10 sample portions are reproduced below:

No. Positives	Most Probable No.	Low Conf. Limit	High Conf. Limit
0	<1.1	-	3.3
1	1.1	0.5	5.9
2	2.2	.37	8.1
3	3.6	.91	9.7
4	5.1	1.6	13
5	6.9	2.5	15
6	9.2	3.3	19
7	12	4.8	24
8	16	5.9	33
9	23	8.1	53
10	>23	12	-

[Para 62] Serial dilutions can be performed in step 102, and steps 103 to 106 can be performed upon each of the dilutions in the series. A most probable number of the specific kind of microorganism in the sample can be determined for each dilution in the series from a table, and the most probable number having the best confidence limits can be selected as the most probable number of the specific kind of microorganism in the sample. Some of the tables in the above-cited Bacteriological Analytical Manual Online, January 2001, Appendix 2, also enable a most probable number to be determined based on the combination of indications from different dilutions in a series.

[Para 63] As discussed above, the most probable number of the specific kind of microorganism determined for the sample can be compared to the number determined for samples of known quantities of the specific microorganism and with control samples known to have none of the specific microorganism. The samples of known quantities and the control samples can confirm that the hybridization and polymerase chain reaction (PCR) techniques are in fact detecting the presence of the specific microorganism in the cultures grown in the most probable number and serial dilution methods.

[Para 64] All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of

the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the methods described herein without departing from the concept and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the scope and concept of the invention.